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PURIFICATION AND AMINO ACID COMPOSITION OF TYPE A BOTULINUM NEUROTOXIN

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B. R. DASGUPTA and V. SATHYAMOORTHY. Purification and amino acid composition of type A botulinum neurotoxin. *Toxicon* 22, 415–424, 1984. — A method to purify type A botulinum neurotoxin from a 64 liter bacterial culture is reported. The procedure includes cation exchange chromatography at pH 7.0. The final product, essentially homogeneous (according to polyacrylamide gel – sodium dodecylsulfate electrophoresis), is a mixture of two forms of the neurotoxin (mol. wt 145,000); the dichain or nicked form (over 95%) and its precursor the single chain or unnicked form. Two batches of the neurotoxin purified by the method described here and one batch purified according to the method of Sugii and Sakaguchi were similar in purity and amino acid composition. The best estimate of the number of amino acid residues per neurotoxin molecule (mol. wt 145,000) is:
$Asp_{200}Thr_{75}Ser_{79}Glu_{114}Pro_{44}Gly_{64}Ala_{53}Val_{70}CyS_{10}Met_{22}Ile_{111}Leu_{104}Tyr_{71}Phe_{68}Lys_{100}His_{14}Arg_{43}Trp_{17}$.

INTRODUCTION

THE ANAEROBIC bacteria *Clostridium botulinum* produces seven serologically distinguishable neurotoxins (types A – G). Among these, type A has been studied most extensively (see references in LAMANNA, 1959; SIMPSON, 1981). This purified protein has not been defined in terms of amino acid composition. Crystallized toxin (LAMANNA *et al.*, 1946; ABRAMS *et al.*, 1946) is a complex of the neurotoxin and a hemagglutinin (see references in LAMANNA, 1959) in a ratio (w/w) of 1:4, respectively (DASGUPTA and BOROFF, 1968). SUGII and SAKAGUCHI (1975) found evidence for three proteins in the crystalline complex. The neurotoxin isolated from the crystalline complex (DASGUPTA and BOROFF, 1967; MOBERG and SUGIYAMA, 1978; TSE *et al.*, 1982) is generally about 95% pure; the contaminants are detectable by SDS-polyacrylamide gel electrophoresis. The neurotoxin isolated from the bacterial culture can also be purified without crystallization (DASGUPTA *et al.*, 1970; SUGII and SAKAGUCHI, 1975; OHISHI and SAKAGUCHI, 1977; TSE *et al.*, 1982).

Reported here are amino acid compositions of the neurotoxin purified according to our method and comparison with the procedure of SUGII and SAKAGUCHI (1975) and OHISHI and SAKAGUCHI (1977).

MATERIALS AND METHODS

Source of the chemicals and materials used were as follows: N-Z Amine type A (Sheffield Farms, Norwich, NY); yeast extract and cooked meat medium (Difco Laboratories, Detroit, MI); autolyzed yeast paste (Yeastamin) (A. E. Staley Manufacturing Co., Decatur, IL); ribonuclease (EC 2.7.7.16; ribonuclease A, Type IA and IIIA) and phenylmethyl sulfonyl fluoride (PMSF) (Sigma Chemical Co., St. Louis, MO); ultrapure guanidine – HCl (Lot # CZ-2777) and ultrapure ammonium sulfate (Schwarz/Mann, Orangeburg, NJ); DEAE-

Sephadex A-50, Sephadex G-100 and SP-Sephadex C-50 (Pharmacia Fine Chemicals). All other chemicals used were of reagent grade. Buffers were prepared with conjugate acid and base of equal molarity titrated to the desired pH at 23 ± 2°C; e.g. 0.05 M citrate buffer pH 5.5 was made with 0.05 M citric acid and 0.05 M trisodium citrate.

Purity of the neurotoxin was assayed (DASGUPTA and SUGIYAMA, 1977) by electrophoresis in 5% polyacrylamide gels in the presence of 0.1% sodium dodecylsulfate (WEBER and OSBORN, 1969). The preparation of samples for electrophoresis and conditions for electrophoretic runs have been previously reported (DASGUPTA and SUGIYAMA, 1972*a*). "Coomassie" dyes used for staining gels were Serva Blue G and Serva Blue R (Serva Fine Biochemicals, Garden City Park, New York). A Gilford linear transport scanner (model 24105) was used for densitometric tracings of gels. Lethality was assayed by i.v. injection of mice (DASGUPTA and SUGIYAMA, 1976). Amount of pure neurotoxin was determined based on 1.63 A_{278} = 1 mg/ml (KNOX *et al.*, 1970*a*). A Gilford spectrophotometer model 260, with a 10 mm light path, was used for all absorbance readings.

Amino acid analysis

Procedures described by MOORE and STEIN (1963) and MOORE (1972) were used for amino acid analysis. Cysteine and cystine were determined as 1/2 cystine (i.e. cysteic acid) following performic acid oxidation and 24 hr hydrolysis in 6 N HCl. Tryptophan was determined from the absorbance of the protein dissolved in 6 M guanidine–HCl (EDELHOCH, 1967). The Durrum D-500 automatic amino acid analyzer was calibrated with standard amino acids (10 nmoles each) immediately before and after analyzing the hydrolyzed protein samples. The exact details of the above procedures which we followed and calculation of the number of amino acid residues have been described (DASGUPTA and RASMUSSEN, 1983*a*). Mol. wt of the neurotoxin, based on the PAGE-SDS technique, is 140,000 (TSE *et al.*, 1982), 145,000 (DASGUPTA and SUGIYAMA, 1972*a*) and 153,000 (OHISHI and SAKAGUCHI, 1977). We used 145,000, the middle value, to calculate the number of amino acid residues.

Bacterial strain

Stock culture of *C. botulinum* type A (Hall strain) (kindly supplied by Dr. Lynn S. Siegel, Fort Detrick, Frederick, MD) in cooked meat medium was maintained in similar medium and stored at 8°C.

Culture medium

Toxin was produced in 5 gallon glass carboys each containing a 16 liter culture. Composition of the medium used was slightly modified from that of SUGIYAMA *et al.* (1977), which was adopted from DUFF *et al.* (1957). A 16 liter solution of N-Z Amine type A 2.0%, yeast extract 0.5%, autolyzed yeast paste 0.6%, adjusted to pH 7.2 with 4 N NaOH, was autoclaved (1 hr, 121°C). To this was added, separately autoclaved (20 min, 121°C) glucose (20% w/v). Final concentration of glucose in the medium was 0.5%. The 16 liter medium was inoculated with 1–2 ml of fluid from the cooked meat medium (stock culture) and incubated without any agitation at 37°C.

Neurotoxin purification

All operations were at room temperature (23 ± 2°C) unless stated otherwise.

Stage 1. After 96 hr incubation the culture was acidified with 3 N H_2SO_4 to pH 3.5. A heavy precipitate settled in 3–4 hr. After siphoning off most of the relatively clear fluid, the precipitate was collected by centrifugation (2400 × *g*, 20 min, 4°C) and washed twice with water. These steps were adopted from the method of DUFF *et al.* (1957).

Stage 2. The precipitate from 64 liters of culture (from 4 carboys) was homogenized and then extracted with ~ 900 ml of 0.1 M citric acid–trisodium citrate buffer, pH 5.5, for 1 hr. The extract was centrifuged (9800 × *g*, 20 min, 4°C) and the pellet re-extracted as before. The deep brown supernatant from the two extractions was pooled, partially saturated with solid ammonium sulfate (35.1 g/100 ml) and held at 8°C at least overnight.

Stage 3. The precipitate, recovered by centrifugation (12,000 × *g*, 20 min, 4°C) was dissolved in ~ 130 ml of 0.05 M Na_2HPO_4–NaH_2PO_4 buffer, pH 6.0, and then digested with ribonuclease (50 μg/ml, 3 hr at 37°C; KOZAKI *et al.*, 1974). The digest (pH dropped as far as 5.5), following centrifugation (23,700 × *g*, 20 min, 23°C) to remove insoluble material, was partially saturated with solid ammonium sulfate (35.1 g/100 ml) and stored at 8° (not more than 16 hr). To minimize proteolytic activity possibly present in ribonuclease, it was dissolved in the pH 6.0 buffer and treated first with 14 μg/ml Trasylol® for 15 min (aprotinin; see ZYZNAR, 1981) and then with 100 μg/ml PMSF for 15 min. The ribonuclease solution containing the two protease inhibitors was used for the digestion step mentioned above.

Stage 4. The precipitate (following ribonuclease digestion) recovered by centrifugation (17,600 × *g*, 20 min, 4°C) was dissolved in ~ 50 ml of 0.05 M citrate buffer, pH 5.5, and then dialyzed against ~ 500 ml of the same buffer for at least 16 hr at 8°C with at least 4 changes of the buffer. During dialysis, the deep brown solution increased in volume to 65–70 ml (due to osmotic pressure difference), a yellow brown pigment came out of the dialysis bag and some material settled at the bottom of the bag. The dialyzed material was held at room temperature for 1 hr and then centrifuged (17,600 × *g*, 15 min, 23°C) to remove the insoluble material. No

more than 17 ml of the deep brown supernatant was loaded on a DEAE-Sephadex A-50 column (2.2 × 45 cm, flow rate 30 – 45 ml/hr) equilibrated with 0.05 M citrate buffer, pH 5.5, and the column was washed with this buffer. Fractions (3 – 5 ml/tube) with an A_{260}/A_{278} ratio of up to 0.6 across the first peak were pooled (a few fractions on the ascending side of the first peak had a ratio higher than 0.6 and these were included). Four such columns were run in parallel to process the toxic material from a 64 liter culture; total protein yield: 257 ml, 2.94 A_{278} to 360 ml, 2.2 A_{278}. The pooled protein solution was incubated with Trasylol and then with PMSF (see end of stage 3) before adding solid ammonium sulfate (35.1 g/100 ml) and storing at 8°C for 16 hr.

Stage 5. The precipitated protein recovered by centrifugation (17,600 × *g*, 15 min, 4°C) was dissolved in ~ 30 ml of 0.05 M citrate buffer, pH 5.5, and then centrifuged (17,600 × *g*, 15 min, 23°C) to remove insoluble material (primarily denatured protein). The supernatant, transparent but slightly yellowish, was fractionated with four parallel Sephadex G-100 columns (2.2 – 2.5 × 60 cm) equilibrated and eluted with 0.05 M citrate buffer, pH 5.5. Each column was loaded with no more than 10.0 ml of sample (storage of this sample at 8° caused turbidity and some precipitation, which was reversed by warming to 23°C). Two very well separated peaks emerged. The larger first peak, containing most of the neurotoxin, from the parallel gel filtration runs was pooled; from a 64 liter culture recovery of total protein was 350 – 370 mg (1.66 A_{278} = 1 mg/ml; KNOX *et al.*, 1970*a*) with an A_{260}/A_{278} ratio of 0.51. The pooled material was treated with Trasylol and PMSF (as in the case of RNase), to inhibit proteolytic activity possibly present, then precipitated with solid ammonium sulfate (39.0 g/100 ml) and stored at 8°C not more than 2 weeks. This is a complex of primarily 2 proteins, the neurotoxin and the hemagglutinin — we refer to it as neurotoxin – hemagglutinin (NT-Hn) complex.

Stage 6. A portion of the precipitated NT-Hn complex, collected by centrifugation (12,000 × *g*, 15 min, 4°C), was dissolved in 0.02 M $Na_2HPO_4 - NaH_2PO_4$, buffer, pH 7.9, and dialyzed sufficiently against this buffer to remove ammonium sulfate. The dialyzed solution was centrifuged (17,600 × *g*, 15 min, 23°C) to remove insoluble denatured protein and then fractionated with a DEAE-Sephadex A-50 column (Fig. 1a). The neurotoxin emerged as the first peak under the linear gradient of increasing Cl^-. Recovery of neurotoxin (1.63 A_{278} = 1 mg/ml) from the NT-Hn complex (1.66 A_{278} = 1 mg/ml) was 21 – 25%.

Stage 7. The neurotoxin was dialyzed against 0.02 M $Na_2HPO_4 - NaH_2PO_4$ buffer, pH 7.0, and loaded on a SP-Sephadex C-50 column equilibrated with the pH 7.0 buffer. What eluted from this column during sample application and column wash (Fig. 1b) was a contaminant that had coeluted with the neurotoxin from the DEAE-Sephadex column. The neurotoxin, bound to the SP-Sephadex column, was recovered as a single peak

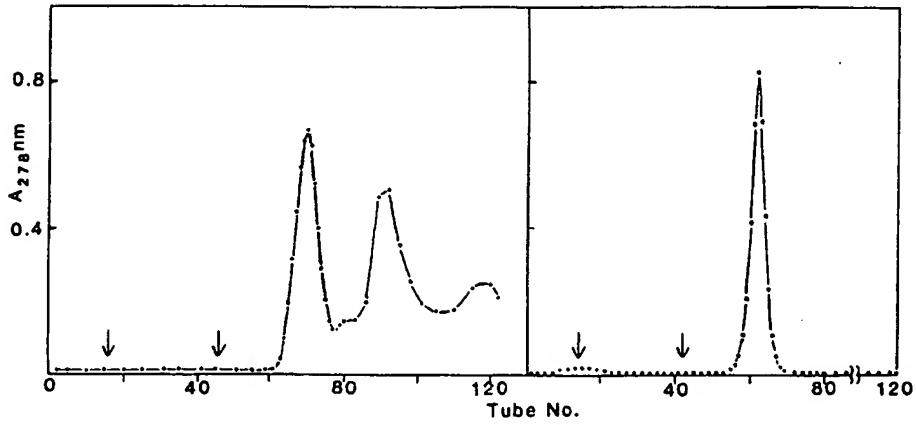

FIG. 1. CHROMATOGRAPHY OF THE NEUROTOXIC MATERIAL.

a. (Left panel) DEAE-Sephadex A-50 column (1.5 × 27.0 cm) equilibrated with 0.02 M Na phosphate buffer, pH 7.9. Sample load 58.0 ml of 1.45 A_{278} and 0.74 A_{260}. Flow rate 20.0 ml/hr. Fraction size 4.0 ml/tube . Arrows indicate beginning of column wash (tube 16) and linear gradient elution at tube 46 (150.0 ml equilibrating buffer plus 150.0 ml equilibrating buffer containing 0.5 M NaCl). Neurotoxin was recovered from tubes 64 – 74; pool 44.0 ml, 0.412 A_{278} and 0.210 A_{260}.

b. (Right panel) SP-Sephadex C-50 column (1.5 × 27.0 cm) equilibrated with 0.02 M Na phosphate buffer, pH 7.0. Sample load 44.0 ml of 0.412 A_{278}. Flow rate 20.0 ml/hr. Fraction size 4.0 ml/tube. Arrows indicate beginning of column wash (tube 14) and linear gradient elution at tube 42 (150.0 ml equilibrating buffer plus 150.0 ml equilibrating buffer containing 0.5 M NaCl). Neurotoxin was recovered from tubes 57 – 66; pool 40.0 ml, 0.360 A_{278} and 0.179 A_{260}.

under a linear gradient of increasing Na^+. Recovery of the neurotoxin from the SP-Sephadex column was ~ 80%.

One batch of NT-Hn complex was isolated from a 30 liter culture according to the method of SUGII and SAKAGUCHI (1975) and the neurotoxin was purified (OHISHI and SAKAGUCHI, 1977) from the complex. The neurotoxin was then subjected to SP-Sephadex chromatography as mentioned above. Final yield of neurotoxin was 24 mg. Two minor changes were made in the procedure; composition of the medium used was as described here and incubation was for 5 days at 37° (not 30°).

RESULTS

Sixty four liters of culture yielded 350 – 370 mg NT-Hn complex (post Sephadex G-100 column) and 60 – 70 mg neurotoxin (post SP-Sephadex column). Specific lethality of the pure neurotoxin was similar to that previously reported (DASGUPTA and SUGIYAMA, 1977). It produced a single precipitin line in Ouchterlony gel diffusion test (1.5% Agar Noble, 0.05 M $KH_2PO_4 - Na_2HPO_4$ buffer, pH 7.5, containing 0.85% NaCl) against antineurotoxin rabbit serum (DASGUPTA and RASMUSSEN, 1981). The neurotoxin migrated as a single band in PAGE-SDS (Fig. 2, left gel) with a 145,000 mol. wt (DASGUPTA and SUGIYAMA, 1972*a*). When the disulfide bond(s) of the neurotoxin was broken with mercaptoethanol (DASGUPTA and SUGIYAMA, 1972*a*) three bands appeared (Fig. 2, right gel). The two prominent bands were the heavy and light chain (mol. wt 97,000 and 53,000, respectively; DASGUPTA and SUGIYAMA, 1972*a*) while the slowest and the least prominent band (mol. wt 145,000) represented the unnicked neurotoxin. The preparation is thus a mixture of dichain and a trace of single chain molecules (see Discussion). When the neurotoxin was not reduced with mercaptoethanol, an additional band, generally very faint, was seen immediately ahead of the neurotoxin (Fig. 2, left gel). The amount of this extra material relative to the neurotoxin was revealed by densitometric scanning of the polyacrylamide gels (see Fig. 2 and Discussion).

Three batches of neurotoxin were analyzed for their amino acid composition. Amino acids recovered from one of these batches following 24, 48 and 72 hr HCl hydrolysis, performic acid oxidation – HCl hydrolysis and the calculated ratio of tryptophan to tyrosine are in Table 1. No unusual peak was found on any of the chromatograms. The calculated number of amino acid residues per neurotoxin molecule (mol. wt 145,000) from Batch #1, 3 and 7 are given in Table 2. There is no consistent difference between Batch 1 and 7 prepared by our method and Batch #3 purified according to SUGII and SAKAGUCHI (1975) and OHISHI and SAKAGUCHI (1977). In those cases where one batch yielded significantly high or low values (i.e. beyond the instrument error), very good agreement was found between the other two batches (the last column in Table 2).

DISCUSSION

The neurotoxin purified by the method reported here and that purified by the method of SUGII and SAKAGUCHI (1975) were comparable in purity and similar in amino acid composition. The type A neurotoxin purified by two different methods has not previously been compared under identical conditions. This observation, seemingly trivial, is indeed significant, because the literature on botulinum neurotoxin includes claims of purification and characterizations (e.g. Gerwing *et al.*, 1965) that are irreproducible (KNOX *et al.*, 1970*b*; DASGUPTA and SUGIYAMA, 1977) Also, previous publications on amino acid composition of type A (BUEHLER *et al.*, 1947; ALSTYNE *et al.*, 1966; STEFANYE *et al.*, 1967; BOROFF *et al.*, 1970) are of little significance because the 1947 and 1967 reports were studies of the crystalline complex (1 part neurotoxin and 4 parts hemagglutinin) and the

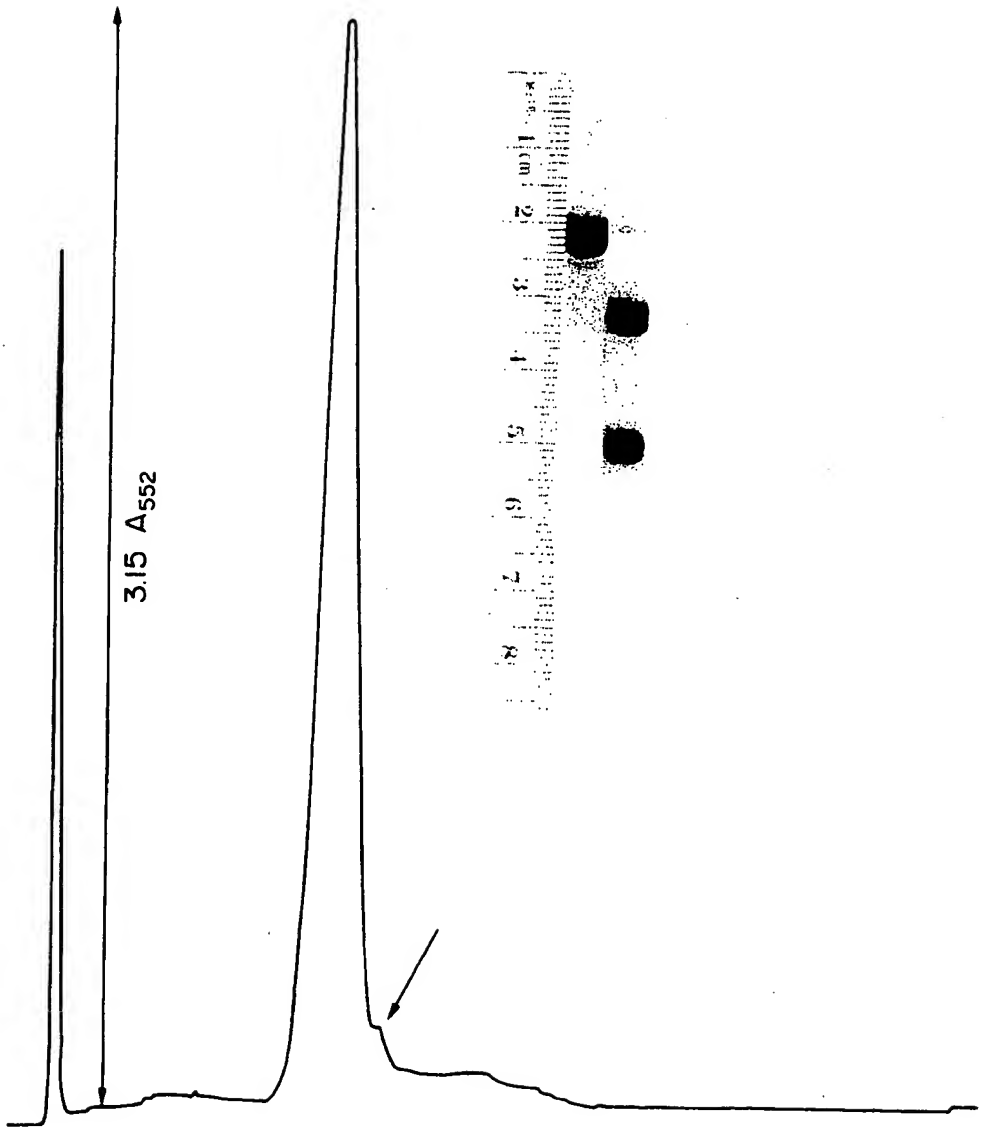

FIG. 2. PAGE-SDS OF TYPE A-NT (UNREDUCED AND REDUCED) AND DENSITOMETRIC TRACING OF A GEL.

Migration in the gel (5%, 5 hr, 8 mA/gel) is from top to bottom (anode). Left gel: neurotoxin not reduced; the thick band is neurotoxin (unnicked and nicked forms). Right gel: neurotoxin reduced with mercaptoethanol; bands between 20–21, 30–35 and 48–53 mm are unnicked neurotoxin and the heavy and light chains, respectively; the sum of the heavy and light chains represents the amount of nicked neurotoxin. The faint band in the left gel between 25 and 26 mm corresponds to the hump (marked with arrow) in the tracing. Full scale = 3.15 A_{552}, chart speed = 4 cm/min, slit plate = 0.2 × 2.36, gel scan = 2 cm/min.

TABLE 1. AMINO ACID ANALYSIS OF BOTULINUM NEUROTOXIN TYPE A (BATCH #1)

	HCl hydrolysis				Mean	Minimum number of residues
	Performic acid*	24 hr	48 hr	72 hr	(Lys = 100)	(His = 1)
Aspartic acid		41.72	45.82	38.80	207.07	17.30
Threonine		14.79	16.04	13.38	76.80†	6.42
Serine		15.73	16.12	13.38	83.50†	6.98
Glutamic acid		24.37	26.79	22.53	120.76	10.09
Proline		8.09	8.76	7.65	40.18	3.36
Glycine	38.47	13.46	14.92	12.70	67.33	5.62
Alanine	32.39	11.11	12.32	10.51	55.63	4.65
Valine	37.90	13.20	15.53	13.95	73.65‡	6.15
Cystine		0.33	0.32	0.22		
Methionine		4.60	5.09	4.26	22.85	1.91
Isoleucine		18.55	23.06	20.75	109.55§	9.15
Leucine		21.80	23.96	21.62	110.59	9.24
Tyrosine		15.54	17.60	14.69	78.34	6.54
Phenylalanine		14.00	15.78	13.30	70.58	5.90
Ammonia		28.0	32.0	29.0		
Lysine	56.00	19.78	22.30	18.94	100.00	8.35
Histidine		2.32	2.68	2.31	11.97	1.00
Arginine	26.33	9.14	10.27	8.65	45.97	3.84
1/2 Cystine	5.87				10.48‖	0.88
Tryptophan					18.87¶	1.58

Columns under performic acid, 24, 48 and 72 hr: nmoles of amino acids recovered from 40 μl hydrolysate. Mean: mean of 24, 48 and 72 hr hydrolysates (except CyS).

*Most of those amino acids (in this column) not included were off scale.

†Thr and Ser extrapolated to zero time.

‡Val 72 hr value (at 24 and 48 hr, 66.73 and 69.64, respectively); slow release.

§Ile 72 hr value (at 24 and 48 hr, 93.78 and 103.40, respectively); slow release.

‖ In batch #3, duplicate values were 9.89 and 9.62; in batch #7 the value was 10.38.

¶ Trp:Tyr = 0.241 (based on 0.602 A_{280} and 0.351 A_{288}). These ratios in batches #3 and #7 were 0.243 and 0.242, respectively.

protein studied in the 1966 report still remains unidentified (KNOX *et al.*, 1970*b*; DASGUPTA and SUGIYAMA, 1977). Lastly, the purity of the neurotoxin used by BOROFF *et al.* (1970) was not assessed by PAGE-SDS and re-examination of the amino acid analysis data indicated that extensive oxidative degradation of amino acids occurred during hydrolysis.

This study provides, for the first time, a chemical basis with which to compare amino acid composition of type A neurotoxin with antigenically different but pharmacologically similar botulinum neurotoxin type B (DASGUPTA and WOODY, 1984), type C (SYUTO and KUBO, 1981), type E (DASGUPTA and RASMUSSEN 1983*a*) and type F (DASGUPTA and RASMUSSEN 1983*b*). The similarity between types A and E is greater than that between any other two types; five amino acids do not differ by more than 1 residue, e.g. type A/type E — Thr 75/75, Pro 44/45, Tyr 71/70, His 14/15, Trp 17/16. The next best resemblance by this criteria is between types A and B, between types A and F and between types E and C; in each pair 3 amino acids do not differ by more than one residue.

The type A neurotoxin we purified is a mixture of two forms; the dichain (or nicked) and its precursor the single chain (or unnicked). Mild trypsinization (pH 6.0, 30°, 30 min) converted the single chain species to the dichain form, as was the case with type B (DASGUPTA and SUGIYAMA, 1976). The amount of unnicked protein in our preparation (Fig. 2, right gel, faint band between 20 and 21 mm) was considerably less than that found

TABLE 2. NUMBER OF AMINO ACID RESIDUES PER TYPE A BOTULINUM NEUROTOXIN MOLECULE (MOL. WT. 145,000)

	Batch 1	Batch 3	Batch 7	Mean of three	Best of three
Aspartic acid	199	186	201	195	200
Threonine	74	76	70	73	75
Serine	80	87	78	82	79
Glutamic acid	116	112	114	114	114
Proline	39	46	43	43	44
Glycine	65	68	63	65	64
Alanine	54	52	53	53	53
Valine	71	69	65	68	70
Half-cystine	10	10*	10	10	10
Methionine	22	22	22	22	22
Isoleucine	105	113	110†	109	111
Leucine	106	103	114†	108	104
Tyrosine	75	71	71	72	71
Phenylalanine	68	69	68	68	68
Lysine	96	100	100	99	100
Histidine	11	14	14	13	14
Arginine	44	46	43	44	43
Tryptophan	18‡	17‡	17‡	17	17

Each batch of neurotoxin was acid hydrolyzed for 24, 48 and 72 hr.
*Based on duplicate determinations (see legend to Table 1).
†These two values include a 72 hr hydrolysate rerun.
‡See legend to Table 1.
Maximum deviation of number of residues from mean:
⩽±3% Glu, Ala, CyS, Met, Phe, Lys;
>±3% but ⩽±5% Asp, Thr, Gly, Val, Ile, Tyr, Arg;
>±5% Ser, Pro, Leu, His, Trp.
Error on Durrum D-500 analyzer is ±3%.

by OHISHI and SAKAGUCHI (1977). The neurotoxin purified by TSE *et al.* (1982) also appears to be a mixture of unnicked and nicked neurotoxin [a faint band in Fig. 5B, track 2 of TSE *et al.* (1982) and a band, stronger in intensity, in Fig. 1, track E of HAMBLETON *et al.* (1981), moving slower than the heavy chain, corresponds to the unnicked neurotoxin].

A faint band migrating ahead of the neurotoxin was seen by PAGE-SDS analysis (Fig. 2, left gel and densitometric tracing). It may be a contaminant or a product of proteolytic degradation of the neurotoxin. Others have also found this or a similar material in their purified neurotoxin in varying concentrations; not detectable (OHISHI and SAKAGUCHI, 1977) and very faint to significant (MOBERG and SUGIYAMA, 1978; TSE *et al.*, 1982, HAMBLETON *et al.*, 1981). We found this band in neurotoxin (Batch #3) prepared according to the methods of SUGII and SAKAGUCHI (1975) and OHISHI and SAKAGUCHI (1977). We could not remove it completely by rechromatography (using DEAE- or SP-Sephadex). The material appears to be a protein of mol. wt 130,000 (TSE *et al.*, 1982; MOBERG and SUGIYAMA, 1978) with ionic properties (chromatographic behavior) indistinguishable from the neurotoxin. It is not seen in PAGE-SDS if the test sample is reduced to break disulfide band(s) (MOBERG and SUGIYAMA, 1978). It appears to be made of at least two chains held by disulfide bond(s) and not to be a single chain polypeptide. The faint band is probably the dichain neurotoxin molecule from which a fragment (mol. wt $\sim$ 15,000) has been clipped off by a proteolytic enzyme(s) of the bacterial culture. INUKAI (1963) has detected at least 3 proteases in the bacterial culture.

To minimize proteolytic degradation, we kept the pH of the neurotoxin preparation at or below 5.5 through the gel filtration step, except for a short period of digestion with RNase. Two protease inhibitors, PMSF (suggested by Dr. John P. Robinson, Vanderbilt University, Nashville, Tennessee) and Trasylol were used. The two inhibitors were also used to minimize protease activity possibly present as a contaminant in ribonuclease. The divalent metal chelating effect of citrate in citric acid – citrate buffer was taken advantage of to sequester Ca^{2+} and Mg^{2+} that often stabilize proteolytic enzymes. The type A culture produces one such protease with trypsin-like activity (DASGUPTA and SUGIYAMA, 1972*b*), which we removed from the neurotoxin following gel filtration (DASGUPTA, 1971; DASGUPTA and SUGIYAMA, 1972*c*) at Stage 5.

Chromatographic conditions used in Stage 4 (DEAE-Sephadex A-50, 0.05 M citric acid-Na_3 citrate buffer, pH 5.5), first introduced to purify type B botulinum neurotoxin (DASGUPTA and SUGIYAMA, 1976), optimized partitioning of proteins from nucleic acids. The neurotoxin is dissociated from hemagglutinin in Stage 6 with 0.02 M $Na_2HPO_4 - NaH_2PO_4$ buffer, pH 7.9 (DASGUPTA and BOROFF, 1967). Two points are significant about the next and final cation exchange chromatography at pH 7.0 (Fig. 1b): this pH is higher than the isoelectric point of the neurotoxin, which is 6.3 (TSE *et al.*, 1982) or 6.1 (DASGUPTA *et al.*, 1970), and yet the neurotoxin is retained by a column equilibrated with 0.02 M $Na_2HPO_4 - NaH_2PO_4$ buffer, pH 7.0; the trace to significant amount of hemagglutinin protein(s) that elutes with the neurotoxin from the DEAE-Sephadex column (in the preceding step) is not retained by the SP-Sephadex column.

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